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갈근황금황련탕추출물의 아토피피부염 유발 백서에서의 피부 보습, 항균, 밀착연접 회복

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Abstract

Effect of Galgeunhwanggeumhwangryeon-tang Extract on Skin Moisturizing, Antibacterial, and Tight Juntion Recovery in Atopic Dermatitis-induced Mice

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Objective

The purpose of this study was to confirm the effects of Galgeunhwanggeumhwangryeon-tang (PSCG) extract on skin moisturizing, antibacterial, and tight junction recovery in atopic dermatitis-induced mice.

Methods

In this study, we used 4-week-old NC/Nga mice divided into four groups: control (Ctrl), lipid barrier elimination (LBE), dexamethasone (Dx) after lipid barrier elimination (DEX), and PSCG after lipid barrier elimination (PSC). Ten rats were assigned to each treatment group. Three days after drug administration following lipid barrier elimination, ceramide kinase, caspase 14, sodium hydrogen antiporter (NHE), cathelicidin, claudin, and toll-like receptor (TLR)-2 were observed to confirm the restoration of skin moisturizer production, antimicrobial barriers, and tight junctions in the skin barrier.

Results

Ceramide kinase and caspase 14 positive reaction were significantly higher in PSC than in LBE and DEX. Both NHE and cathelicidin showed higher positive reactions in PSC than in LBE and DEX. Claudin, and TLR-2 showed higher levels of positive staining in the PSC group than in the LBE and DEX groups.

Conclusion

It was confirmed that the PSCG extract can have the potential to restore the damaged skin barrier in atopic dermatitis.

Key words: Galgeunhwanggeumhwangryeon-tang, Atopic Dermatitis, Ceramide, Tight junction

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Ⅰ. Introduction

Atopic dermatitis is a chronic inflammatory disease that is frequently associated with conditions such as allergic rhinitis and asthma[1]. Atopic dermatitis has a high prevalence in children and is usually diagnosed by dryness, pruritus, and erythematous eczema[2,3]. Recently, atopic dermatitis has been increasingly seen in adults due to mental stress, environmental pollution, and poor dietary habits[4].

Many patients with atopic dermatitis have symptoms of erythema, edema, exudation, and erosion along with intense itching. The pathogenesis of atopic dermatitis has been variously considered to be genetic, environmental, immunologic, and skin barrier abnormalities[5,6]. This study suggests that ceramide reduction, antibacterial peptide reduction, and tight junction abnormalities associated with skin barrier dysfunction are the main pathogenesis of atopic dermatitis[7]. We wanted to focus on whether these pathogenic factors could be effectively treated with herbal medicine.

In Korean medicine, atopic dermatitis is referred to as fetal dermatitis (胎癬) or nasal dermatitis (奶癬)[8], and also includes eczema or eczema (濕瘡)[9]. Atopic dermatitis is caused by the invasion of wind-heat (風熱) from the outside or the accumulation of damp-heat (濕熱) from the inside without the fetal heat (胎熱)[8]. The main treatment for atopic dermatitis is heat clearing (淸熱), and other treatments are also used to removing the wind (祛風) and nourishing the blood (養血潤燥)[9].

Galgeunhwanggeumhwangryeon-tang (葛根黃芩黃連湯, PSCG) is a prescription consisting of *Puerariae Radix* (葛根), *Scutellaria baicalensis* (黃芩), *Coptidis Rhizoma* (黃連), and *Glycyrrhiza uralensis* (甘草). *Puerariae Radix, Scutellaria baicalensis*, and *Coptidis Rhizoma* are commonly used for inflammatory skin conditions due to their cooling and detoxifying effects. *Puerariae Radix* is known to regulate the inflammatory response of the skin by inhibiting the secretion of interleukin (IL)-3, IL-4, and tumor necrosis factor-alpha (TNF-α) through its antiperspirant, antiphlogistic, and antidote properties[10]. *Scutellaria baicalensis* is used to treat skin boils and leprosy by detoxifying heat and moisturizing heat. More recently, it has been used to treat inflammatory skin conditions such as atopic dermatitis and eczema[11]. It is also known for its anti-inflammatory properties and ability to repair damaged skin barriers[12]. *Coptidis Rhizoma* is effective in suppressing inflammation and repairing skin damage caused by burns, as it has the effects of cooling, moisturizing, and detoxifying, as well as antiviral and antibacterial effects[13,14].

This study aimed to determine whether PSCG extract can alleviate skin epithelial damage, restore skin moisturizing factor production through changes in ceramide kinase and caspase 14, restore antibacterial barrier through changes in sodium hydrogen antiporter (NHE) and cathelicidin, and restore tight junction through changes in claudin and toll-like receptor (TLR)-2.

In this study, we report the results that showed the potential of PSCG extract to restore the impaired skin barrier environment in atopic dermatitis.

Ⅱ. Materials and Methods

1. Materials

1) Animals

Four-week-old male NC/Nga mice were obtained from JA-Bio (Seoul, Korea). To minimize possible subjective bias in evaluating the results, mice were acclimatized for 2 weeks and then selected at a weight of 16 ± 1 g.

Mice in all groups were housed at 23-25 °C, 55 ± 10% humidity, 12 hr light/dark cycle, and provided with a diet of SAFE-40+RMM (SAFE, France) and filtered tap water *ad libitum*. Animal experiments were conducted after approval by the Institutional Animal Care and Use Committee of Semyung University (IACUC No. smecae 20-11-01), and other care and use of laboratory animals was in accordance with the guidelines of the National Institutes of Health (NIH).

2) Preparation of PSCG Extract

PSCG was purchased from Barunhanyak (Seoul, Korea). PSCG [105g (Table 1); *Pueraria lobata* (Willd.) Ohwi (葛根) 52 g, *Scutellaria baicalensis* George (黃芩) 20 g, *Coptis*

japonica Makino (黃連) 13 g, *Glycyrrhiza uralensis* Fischer (甘草) 20 g} was added to 2000 ml of distilled water, steeped for 3 hours, and filtered. The filtrate was reduced to 100 ml using a rotary evaporator (Eyela, Tokyo, Japan), concentrated, and dried with a freeze-dryer (Labconco, Kansas, MO, USA) to obtain 20.4 g of extract (yield 19.4%).

2. Methods

1) Induction of Model and Drug Treatment

The experimental groups were: control (Ctrl), lipid barrier elimination group (LBE), dexamethasone (Dx) administration group after lipid barrier elimination (DEX) and PSCG extract administration group after lipid barrier elimination (PSC). Each group consisted of 10 animals. The dorsal area of the skin of mice was depilated using an electric shaver and depilatory cream (Body natur, Nueil-les-Aubiers, France). The stratum corneum was then removed using tape (3M, St. Paul, MN, USA). To remove the fatty barrier of the stratum corneum, 500 µl of 10% sodium dodecyl sulfate (SDS: Sigma-Aldrich, St. Louis, MO, USA) was treated with a cotton swab 20 times. Aggressive hyperactive and avoidant individuals were excluded from the study by observing their behavior during the experimental period.

A daily dose of 340 mg/kg of PSCG extract was prepared by dissolving it in 0.2 ml of normal saline. It was administered orally for 5 days after removing the fat barrier. Dexamathasone (Sigma-Aldrich), used as a control drug, was also administered orally in the same manner as PSC.

2) Tissue Chemistry

The skin was subjected to vascular rinse and cardiac perfusion fixation in 10% neutral buffered formalin (NBF). The harvested dorsal skin was fixed in 10% neutral buffered formalin for 24 hours at room temperature, then embedded in paraffin and serially sectioned at 5 µm thickness. Histologic structures were observed by Masson's trichrome staining to investigate the changes in atopic dermatitis-induced skin in response to the extract.

3) Immunohistochemistry

Immunohistochemical staining with anti-ceramide kinase K, anti-caspase 14, anti-NHE1, anti-cathelicidin, anti-claudin, and anti-TLR-2 was performed to investigate the immunohistological changes of ceramide kinase K, caspase 14, sodium hydrogen antiporter 1 (NHE1), cathelicidin, claudin, and TLR-2.

Skin sections were first subjected to proteolysis with proteinase K (20 µg/µL; Dako, Santa Clara, CA, USA) for 5 minutes. The sections were then blocked in 10% normal goat serum (Vector Lab, Burlingame, CA, USA) with 1% fetal bovine serum (Sigma-Aldrich) for 1 hour. Primary antibodies (mouse anti-ceramide kinase (1:100, Santa Cruz Biotechnology Inc.), mouse anti-Caspase 14 (1:100, Santa Cruz Biotechnology Inc.), mouse anti-NHE (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse anti-cathelicidin (1:100, Abcam, Waltham, MA, USA), mouse anti-claudin (1 : 100, Santa Cruz Biotechnology Inc.), and mouse anti-TLR-2 (1:100, Abcam, Waltham, MA, USA)) were reacted for 72 hours in a humidified chamber at 4 °C. The secondary antibody, biotinylated goat anti-mouse Immunoglobulin (Ig) G (1:50, Santa Cruz Biotechnology Inc.), was linked for 24 hr at room temperature and reacted with avidin biotin complex kit (Vector Lab) for 1 hr at room temperature. The blots were developed in 0.05 M tris-HCl buffer (pH 7.4) containing 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen chloride (HCl) and counterstained with hematoxylin.

4) Image Analysis

The results of immunohistochemistry were quantified (means ± standard error) by image analysis using image Pro 10 (Media cybernetics, Rockville, MD, USA). Ten skin samples from each group were randomly selected and photographed at x 100 magnification and analyzed as positive pixels (intensity 80~100) / 20,000,000 pixels.

5) Statistical Analysis

For statistical analysis, the experimental data were analyzed using SPSS software (SPSS 25, SPSS Inc., Chicago, IL, USA). The statistically significant differences were verified using one way analysis of variance (ANOVA) and

Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

Ⅲ. Results

1. PSCG Reduces Skin Epithelial Damage

In LBE and DEX, dilated intercellular spaces were seen in the visible layer of the injured dermal epithelium, and structural changes with increased lymphocyte infiltration were observed around the base of the collapse in the basal lamina. Dermatitis damage was reduced in PSC compared to LBE and DEX (Fig. 1).

2. PSCG Restores Skin Moisturizer Production

Ceramide kinase positivity was observed in the stratum corneum and granular layer. Ceramide kinase positivity was increased in LBE (22,885 ± 735/20,000,000 pixels), DEX (33,234 ± 702/20,000,000 pixels), and PSC (50,077 ± 717/20,000,000 pixels) compared to Ctrl (10,539 ± 421/20,000,000 pixels). LBE increased by 117% compared to Ctrl, DEX increased by 215%, and PSC increased by 375%. Ceramide kinase positivity in PSC was significantly increased by 119% compared to LBE and 51% compared to DEX (Fig. 2).

Caspase 14 positivity was observed in the granulation layer. Caspase 14 positivity was decreased in LBE (11,193 ± 375/20,000,000 pixels) and increased in DEX (24,937 ± 462/20,000,000 pixels) and PSC (39,750 ± 825/20,000,000 pixels) compared to Ctrl (19,740 ± 408/20,000,000 pixels). LBE decreased by 43%, DEX increased by 26%, and PSC increased by 101% compared to Ctrl. Caspase 14 positivity in PSC significantly increased by 255% compared to LBE and 59% compared to DEX (Fig. 2).

3. PSCG Restores the Antimicrobial Barrier

NHE positivity was observed in the stratum corneum and the granular layer. NHE positivity decreased in LBE (16,094 ± 453/20,000,000 pixels) compared to Ctrl (11,527 ± 580/20,000,000 pixels), and increased in DEX (17,713 ± 429/20,000,000 pixels) and PSC (26,186 ± 547/20,000,000 pixels). LBE decreased by 28% compared to Control, while DEX and PSC increased by 10% and 63%, respectively, compared to Control. NHE positivity in PSCs was significantly increased by 127% compared to LBE and 48% compared to DEX (Fig. 3).

Cathelicidin positivity was observed in the stratum corneum and granular layer. Cathelicidin positivity decreased in LBE (12,288 ± 566/20,000,000 pixels) and DEX (20,009 ± 566/20,000,000 pixels) compared to Ctrl (34,395 ± 585/20,000,000 pixels), and increased in PSC (55,250 ± 1,061/20,000,000 pixels). Compared to control, LBE decreased by 64% and DEX decreased by 42%, while PSC increased by 61% compared to control. Cathelicidin positivity in PSC significantly increased by 350% compared to LBE and 176% compared to DEX (Fig. 3).

4. PSCG Restores Tight Junction

Cathelicidin positivity was observed in the stratum corneum and granular layer. Claudin positivity was reduced in both LBE (13,076 ± 531/20,000,000 pixels), DEX (18,127 ± 279/20,000,000 pixels), and PSC (26,205 ± 280/20,000,000 pixels) compared to Ctrl (30,489 ±

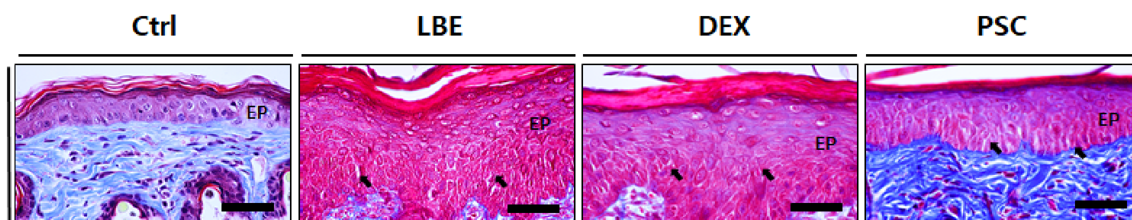

Figure 1. The mitigative effects of skin lesions by Galgeunhwanggeumhwangryeon-tang (PSCG) extract treatment

The PSCG extract treatment relieved symptom as enlargement of intercellular space (arrow) in PSC compared with LBE and DEX. Abbreviations. Ctrl, normal; LBE, lipid barrier eliminated group; DEX, dexamethasone (Dx) administration group after lipid barrier elimination; PSC, PSCG extract administration group after lipid barrier elimination; M/T, Masson's trichrome; EP, epithelium; Arow, intercellular space: Bar size, 50㎛.

531/20,000,000 pixels). Compared to Ctrl, LBE was reduced by 57%, DEX by 41%, and PSC by 14%. Claudin positivity in PSCs was significantly increased by 100% compared to LBE and 45% compared to DEX (Fig. 3).

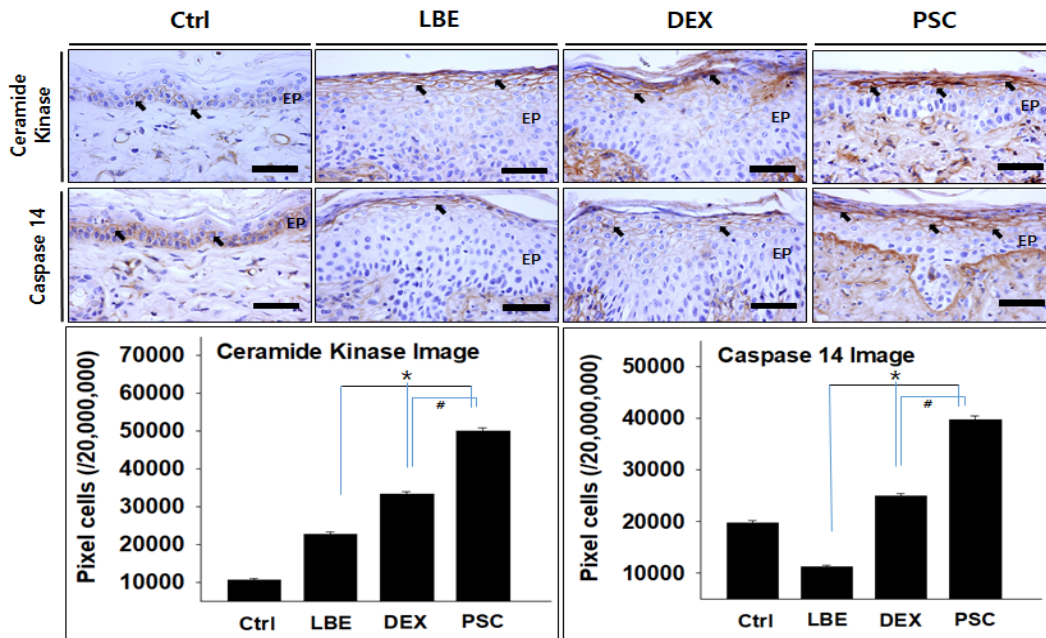

Figure 2. The regeneration of normal moisturizing factor by PSCG extract treatment (ceramide kinase and caspase 14 immunohistochemistry)

The activation of ceramide kinase and caspase 14 (arrow indicates light brown particle) was significantly increased in PSC as compared with LBE and DEX, the data of ceramide kinase and caspase 14 image analysis showed the same results. (*, $p < 0.05$ compared with LBE; #, $p < 0.05$ compared with DEX) Abbreviations same as Fig. 1.

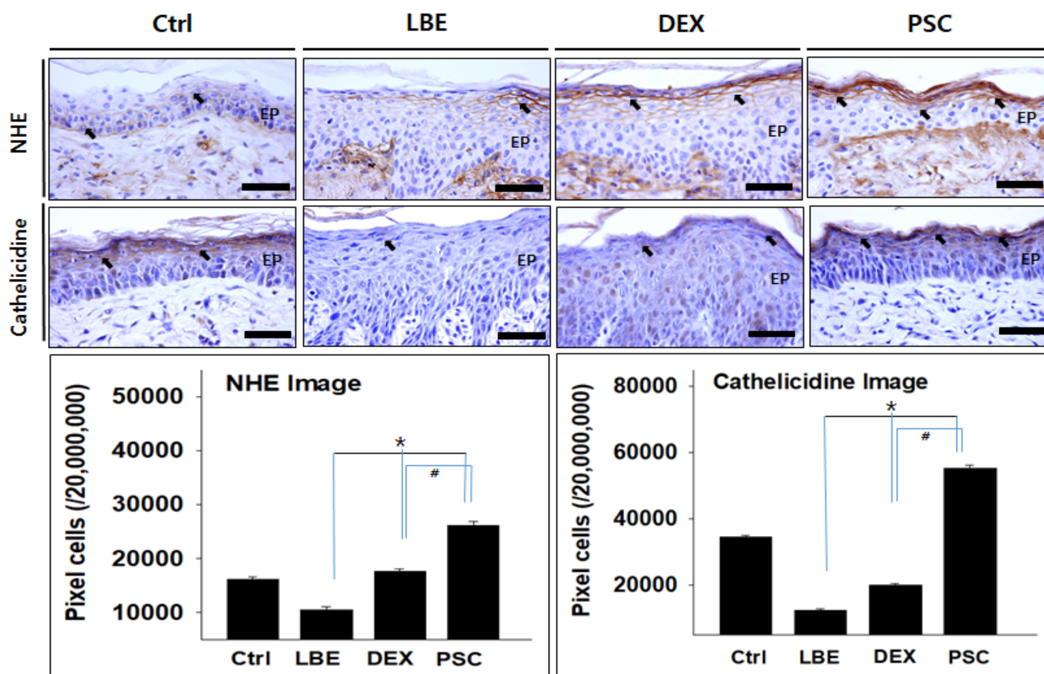

Figure 3. The regenation of anti-microbial barrier by PSCG extract treatment (NHE and cathelicidin immunohistochemistry)

The activation of sodium hydrogen antiporter (NHE) & cathelicidin (arrow indicates light brown particle) was significantly increased in PSC as compared with LBE and DEX, the data of NHE and cathelicidin image analysis showed the same results. (*, $p < 0.05$ compared with LBE; #, $p < 0.05$ compared with DEX) Abbreviations same as Fig. 1.

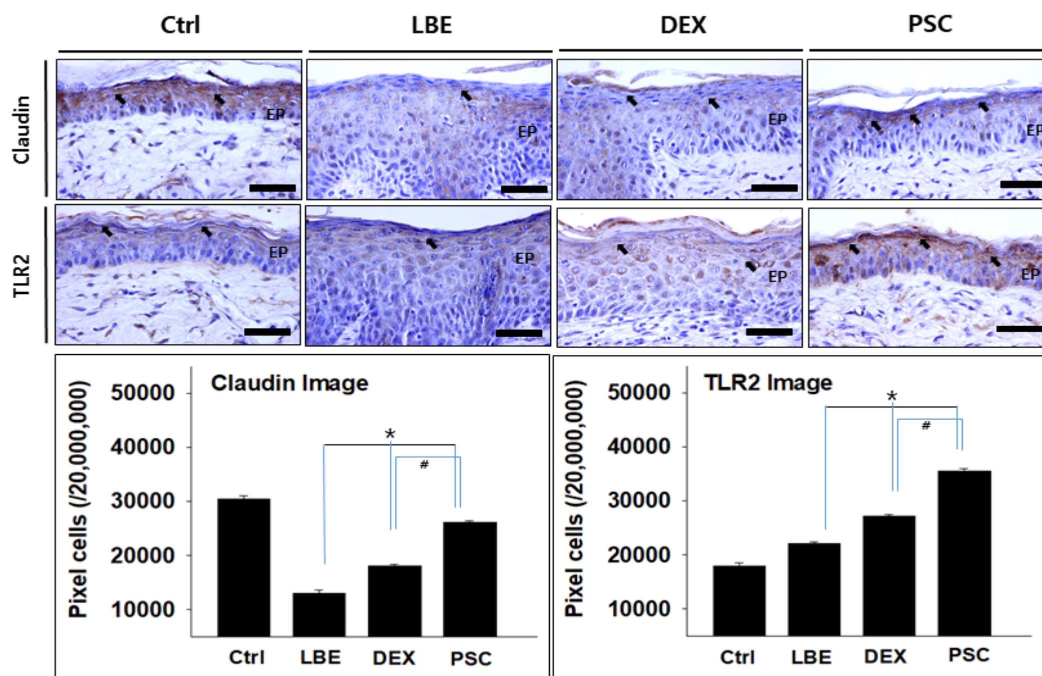

Figure 4. The regeneration of tight junction by PSCG extract treatment (claudin and TLR-2 immunohistochemistry)

The activation of claudin and toll-like receptor (TLR)-2 (arrow indicates light brown particle) was significantly increased in PSC as compared with LBE and DEX, the data of claudin and TLR-2 image analysis showed the same results. (*, $p < 0.05$ compared with LBE; #, $p < 0.05$ compared with DEX) Abbreviations same as Fig. 1.

TLR-2 positivity was also observed in the granular layer. TLR-2 positivity was increased in LBE (22,070 ± 390/20,000,000 pixels), DEX (27,103 ± 414/20,000,000 pixels), and PSC (35,458 ± 414/20,000,000 pixels) compared to Ctrl (17,943 ± 540/ 20,000,000 pixels). Compared to Ctrl, LBE increased by 23%, DEX by 51%, and PSC by 98%. TLR-2 positivity in PSC significantly increased by 61% compared to LBE and 31% compared to DEX (Fig. 4).

Ⅳ. Discussion

Atopic dermatitis is a typical inflammatory skin disease caused by skin barrier dysfunction. In Korean medicine, it has been recognized as a febrile condition caused by fetal heat (胎熱)[8].

There are various pathogenic factors associated with skin barrier dysfunction in atopic dermatitis[15]. Among them are decreased ceramides in the stratum corneum[16], decreased antimicrobial peptides[17], decreased serine proteases inhibitors[18], tight junction abnormalities[19], and filaggrin gene (FLG) mutations[20].

Patients with atopic dermatitis have a marked decrease in ceramides, a major component of the lipids between keratinocytes in the stratum corneum. Ceramides are the main water-retaining molecules between the skin's keratinocytes[21]. This decrease in ceramides, along with a decrease in skin moisturizing factor, a breakdown product of filaggrin, is the primary cause of the dryness observed in almost all atopic dermatitis patients[22]. In normal skin, filaggrin is present in keratinocyte granules as the precursor profilaggrin, which is then degraded by proteases to become filaggrin. Filaggrin holds keratin fibers together and forms the physical support of the skin barrier[23]. When filaggrin is deaminoacidified, the adhesion between keratin fibers decreases, and the isolated filaggrin units are degraded by caspase14, calpain, bleomycin hydrolase, etc. into amino acids such as pyrrocarboxylic acid (PCA) and trans-urocanic acid (UCA), which are natural moisturizing factors and maintain skin moisture[24]. The findings that ceramide kinase and caspase 14 positivity of PSC were significantly increased compared to LBE and DEX

suggest the possibility of restoring skin moisturizing function impaired by atopic dermatitis.

The antimicrobial barrier function of the skin is maintained by a combination of the low pH of the skin surface, the lipid barrier function between skin keratinocytes, and antimicrobial peptides in the stratum corneum. Among these, antimicrobial peptides are one of the important components of the innate immune system and are an important component. Antimicrobial peptides secreted by the skin include cathelicidin, elafin, psoriasin, dermcidin, lysozome, ribonuclease (RNase) 7, and adrenomedullin[25]. These antimicrobial peptides are secreted from the lamellar plasma membrane into the stratum corneum, where they remain low in normal skin conditions and increase dramatically when wounds or infections occur, increasing the antimicrobial capacity of the skin[26]. In patients with atopic dermatitis, the expression of antimicrobial peptides is reduced, resulting in a dysfunctional antimicrobial barrier[27]. The significant increase in cathelicidin positivity of PSC by 350% compared to LBE and 176% compared to DEX shows that PSCG extract has the potential to restore antimicrobial barrier function by increasing antimicrobial peptide expression.

In atopic dermatitis, when the skin barrier is compromised, tight junction proteins provide an additional barrier against allergen entry due to stratum corneum damage. In a recent study, claudin-1 and claudin-23 were found to be reduced in patients with atopic dermatitis[19]. In this study, claudin positivity of PSC was significantly increased compared to LBE and DEX, suggesting that PSCG extract may enhance adhesion by increasing claudin.

Innate immune receptors are expressed on keratinocytes and antigen-presenting cells in the skin, called pattern recognition receptors. Among these, toll like receptors (TLRs) are generally the most widely recognized[28]. When the skin barrier is compromised or bacteria invade from outside, TLRs are stimulated to release antimicrobial peptides, cytokines, and chemokines. This strengthens the barrier and prevents further bacterial invasion[29]. In patients with atopic dermatitis, the function of TLRs is decreased, leading to increased serum IgE and increased *S. aureus* infection. In addition, increased Th2 cytokines decrease the expression of antimicrobial peptides in the skin, making it vulnerable to bacterial and viral infections[28]. The results of our study confirmed that PSCG extract increases TLRs. This is expected to restore the decreased TLR function in patients with atopic dermatitis, thereby strengthening the hermetic seal and reducing bacterial and viral infections.

We found that PSCG extract has the potential to treat atopic dermatitis by restoring the damaged skin barrier environment. However, this study is limited to animal experimental studies. It is difficult to directly apply the results of this study to clinical practice. It can be seen as a suggestion of the potential of PSCG extract to treat atopic dermatitis. We look forward to further clinical studies to establish clinical evidence based on this study.

Ⅴ. Conclusion

This study observed that PSCG extract alleviated skin epithelial damage, restored skin moisturizing factor production through changes in ceramide kinase and caspase 14, restored antibacterial barrier through changes in NHE and cathelicidin, and restored adhesion through changes in claudin and TLR-2. The following results were obtained.

1. Histochemical observation of injured dermal epithelium showed that PSC reduced dermatitis damage compared to LBE and DEX.
2. Ceramide kinase positivity was significantly increased by 119% in PSC compared to LBE and 51% compared to DEX.
3. Caspase 14 positivity was significantly increased by 255% in PSC compared to LBE and 59% compared to DEX.
4. NHE positivity was significantly increased by 127% in PSCs compared to LBE and 48% compared to DEX.
5. Cathelicidin positivity was significantly increased by 350% in PSCs compared to LBE and 176% compared to DEX.

6. Claudin positivity was significantly increased by 100% in PSCs compared to LBE and 45% compared to DEX.
7. TLR-2 positivity was significantly increased by 61% in PSCs compared to LBE and 31% compared to DEX.

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